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DNA Block Copolymer Micelles – A Combinatorial Tool for Cancer Nanotechnology**

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Selective drug targeting of a specific organ or tissue is a challenging task. This holds especially true for chemotherapeutic cancer treatment because most of the available anticancer agents cannot distinguish between cancerous and healthy cells, leading to systemic toxicity and undesirable side effects. One effective approach to address this problem is the application of polymeric nanoparticles equipped with targeting units for tumor-specific delivery.[1] For instance dendrimers, highly branched macromolecules, can be equipped with targeting units as well as with anticancer drugs because of their large number of surface functionalities.[2] Amphiphilic block copolymers, which self-assemble in dilute aqueous solutions into three-dimensional spherical micelles with a hydrophilic corona and a hydrophobic core, are another attractive option. These nanosized objects, with a typical size of 10–100 nm, are able to accommodate lipophilic drugs in their interior and alter their kinetics in vitro and in vivo.[3] Different polymeric systems such as shell crosslinked nanoparticles (SCKs),[4] poly(D,L-lactic-*co*-glycolic acid)-*b*-poly(ethylene glycol),[5] poly(ethylene glycol-*b*-ε-caprolactone)[6] block copolymers and poly(*N*-isopropylacrylamide acrylic acid) microgels[7] have also been successfully utilized in combination with targeting units.

Folate receptors (FRs), which are highly expressed on the surface of various cancer cells, emerged as new targets for specific localization of chemotherapeutics incorporated into nanoparticle systems. The family of FRs currently consists of three known isoforms: FRα, FRβ and FRγ.[8] FRα is expressed primarily in cancer cells such as ovarian, testicular, breast, colon, renal and malignant nasopharyngeal carcinomas.[9–12] The process that mediates targeting of the folate-linked nanoparticle to the receptor and subsequent internalization is identical to that for the free folate.[1] As reviewed by Reddy et al., folates, after binding to their receptors, are taken up by the cells via the receptor-mediated endocytic pathway.[13]

Recently, a new type of amphiphilic block copolymer has emerged that is composed of a hydrophobic synthetic polymer component and a biological segment consisting of an oligodeoxynucleotide (ODN) sequence.[14–16] Micelles composed of these materials exhibit a corona of single-stranded (ss) DNA and have been utilized for the delivery of antisense ODNs,[17] for the hybridization with DNA-coated gold nanoparticles[18] and as programmable, three-dimensional (3D) scaffolds for DNA-templated organic reactions.[19]

Here, we introduce DNA block copolymer micelles as a highly modular system for chemotherapeutic drug delivery. ODN-modified targeting units were "clicked" into the micelle corona by hybridization, allowing perfect control of surface functionalities of the nanoparticle system. The interior of the micelles was loaded efficiently with a hydrophobic anticancer drug. Cell culture experiments revealed that cellular uptake strongly depends on the density of targeting units on the surface of the carriers. As a result, cancer cells were efficiently killed when targeting units and chemotherapeutic acted together within the DNA block copolymer drug delivery system (Fig. 1).

Polypropylene oxide (PPO) was selected as the hydrophobic component of the DNA block copolymer to provide a polymer with proven biocompatibility toward different cell types when administered as a constituent component of amphiphilic block copolymer micelles.[20] For the generation of the DNA-*b*-PPO copolymer, a phosphoramidite-functionalized PPO (M_n=6800 g mol^{-1}) was synthesized and attached to the 5' terminus of the nucleic acid fragment (5'-CCTCGCTCTGCTAATCCTGTTA-3', 22mer, M_w= 6700 g mol^{-1}) via automated solid phase synthesis as reported previously.[19] The resulting block copolymer was analyzed and purified by denaturing polyacrylamide gel electrophoresis (PAGE) and the molecular weight was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Supporting Information, SI). Dynamic Light Scattering (DLS) measurements of the DNA

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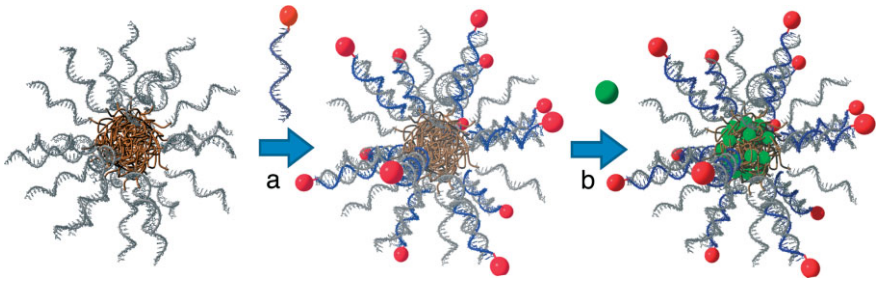

Figure 1. Schematic representation of the drug delivery system based on DNA block copolymers. a) Targeting units (red dots) that are connected to the complementary sequence of the micelles are hybridized to equip the nanoparticle surface with FA units. b) The anticancer drug (green dots) is loaded into the core of the micelles. Due to hydrophobic interactions of Dox with PPO the drug accumulates in the interior of the block copolymer aggregates.

block copolymer aggregates revealed the formation of uniform micelles of diameter (10.8±2.2) nm consistent with previous findings.[16,19] To equip these micelles with targeting units, 5'- and 3'-amino-modified ODNs that encode the complementary sequence of DNA-*b*-PPO were reacted with folic acid (FA) in the presence of DMT-MM 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride.[21] After purification by PAGE the folic acid-functionalized ODNs were obtained in 65 % yield. These conjugates can be hybridized with the micelles so that the FA is either positioned at the periphery (5') (Fig. 1a) or in the core (3') of the nanoparticle.

In order to study the effect of FA density and position within the nanoparticles on the targeting efficiency, DNA-*b*-PPO copolymers were hybridized in different ratios with the targeting unit-bearing ODNs. This convenient procedure resulted in micelles with on average 2, 11, or 28 (fully hybridized) FAs either at the periphery or at the hydrophobic–hydrophilic interface of the micelles. The dimensions of the micelles were again assessed by DLS, which revealed maintenance of their narrow size distribution. Moreover, the diameter of the micelles was found to increase slightly with an increase in the number of FA units. For 2, 11, and 28 FA moieties at the rim, micelle diameters of (11.2±1.6) nm, (13.2±2.4) nm, and (14.4±2.2) nm were measured, respectively. When FA is positioned inside, diameters of (11.2±1.8) nm, (12.2±2.4) nm, and (12.2±2.0) nm were detected for the same FA densities. Importantly, the nanoparticles were of the order of 10 nm, an important design criterion for efficient tumor cell-specific delivery.[22] Although it has been proven that polymer particles in the range of 100 nm exit the vasculature and enter tumor tissue through a process known as the enhanced permeability and retention (EPR) effect,[23–25] it is in some cases favorable to make use of delivery independent of fenestrate pore cutoff size. This can occur when particles have a diameter of less than 10 nm, as albumin molecules do.[26] This is supported by recent computer simulations of cancer progression at the tumoral level.[27] It was demonstrated that nanoparticles with a size range of 1–10 nm diffuse directly and target the individual cell, which results in improved tumor response.

Human colon adenocarcinoma (Caco-2) cells were employed as a cancerous cell line to study the uptake of the differently decorated DNA block copolymer micelles since they have already been used as a model to study nanoparticle uptake.[28] Moreover, their FA uptake has been characterized previously.[29] In the present study the availability of three known genes for folic acid transport, that is, RFC, FRα and FRβ, were examined and their relative gene expression levels were measured by real-time polymerase chain reaction (PCR) (see SI). Quantitative real time PCR has become the most prevalent method for quantification of mRNA transcription levels due to its outstanding accuracy, broad dynamic range and sensitivity.[30] According to PCR experiments the three analyzed genes are expressed at different levels. The Caco-2 cells express a high level of FRα, which is consistent with previous findings.[8,31] It should be added here that there was no apparent difference between the older and the younger passage, suggesting no loss of expression of the transporter genes by further splitting. FRα is also highly expressed in other solid epithelial tumors such as ovarian carcinoma and mesothelioma. Thus this cell line is well suited to act as a model to study the effect of targeting cancerous cells.

Before analyzing the uptake of DNA block copolymer micelles, we assessed their biocompatibility. In vitro cytotoxicity was determined based on an XTT cell proliferation assay 2,3-bis[2-methoxy-4-nitro-5sulfenyl]-2H-tetrazolium-5-carboxanclid. The Caco-2 cells were incubated with different concentrations of DNA-*b*-PPO copolymer and of their FA-functionalized derivatives. Toxicity of the DNA block copolymer was quantified spectrophotometrically at 450 nm and revealed that more than 75 % of the cells were viable (see SI for the viability of each nanoparticle). Motivated by the nontoxic nature of the nanoparticles, we proceeded to study their uptake into Caco-2 cells. For tracking purposes 4 % of the nanoparticles were additionally labeled with a fluorescent dye. PPO-*b*-DNA micelles were hybridized with 3'-Alexa488-functionalized oligonucleotides encoding the complementary sequence of the DNA corona so that the dye was located in the interior. These micelles were then administered to the folate-receptor-bearing Caco-2 cells (see SI for experimental details). The internalization of the micelles was determined by confocal laser scanning microscopy (CLSM) and, after lysing the cells, by fluorescence spectroscopy. The latter method offers the possibility to quantitatively compare the uptake of nanoparticles. As shown in Figure 2a, an increasing number of FA entities at the surface of the micelles strongly promoted internalization. With only 2 targeting units present the uptake into the cells was comparable to nonfunctionalized DNA-block-copolymer micelles. When the average number of targeting units was adjusted to 28, the uptake increased by a factor of 10 compared

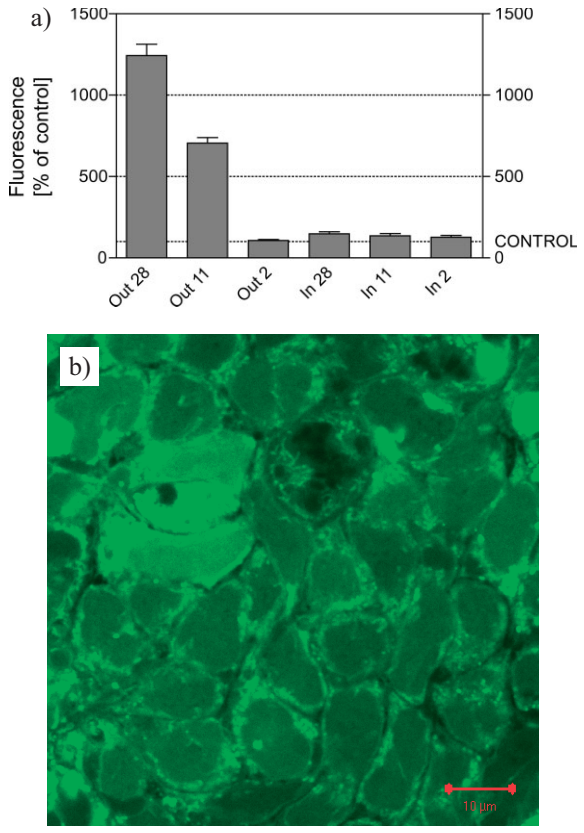

Figure 2. a) Uptake of folic acid linked micelles into human Caco-2 monolayers incubated for 3 h. Out 28: DNA-block-copolymer micelles with 28 targeting units at the periphery of the micelle, Out 11: DNA-block-copolymer micelles with 11 targeting units at the periphery of the micelle, Out 2: DNA-block-copolymer micelles with 2 targeting units at the periphery of the micelle, In 28: DNA-block-copolymer micelles with 28 targeting units in the core of the micelle, In 11: DNA-block-copolymer micelles with 11 targeting units in the core of the micelle and In 2: DNA-block-copolymer micelles with 2 targeting units in the core of the micelle. Results are shown as the average values of triplicates ± standard deviation (SD). b) CLSM image of the uptake of labeled micelles inside Caco-2 cells.

to the control. In contrast, when the targeting moieties pointed towards the interior of the micelles the uptake was comparable with bare DNA-*b*-PPO aggregates. From these experiments three important conclusions can be drawn. The uptake of DNA-block-copolymer micelles strongly depends on the number of targeting units at the rim. Furthermore, the higher the number of FA entities, the more efficiently the nanoparticles are internalized. Finally, when the targeting units are hidden inside the nanoparticles they cannot be "recognized" by the folate receptors, indicating that the micelles remain intact and do not dissociate into isolated block copolymers.

CLSM has proven to be a powerful tool for acquiring high-resolution images, 3D reconstructions and visualizations of internalization of nanoparticles.[32–35] Figure 2b shows the CLSM image of Caco-2 cells after 3 h incubation with DNA-block-copolymer micelles labeled with 28 targeting units at the surface that exhibited the most efficient uptake. 3D slicing experiments showed that the nanoparticles were internalized homogenously and did not only adsorb on the membrane (data not shown). No distinct patterns of subcellular staining were observed. It must be pointed out that the incubation experiments were performed in Hanks buffer, which does not contain any protein that may interact with the nanoparticles.

After the optimization of the targeting properties of the nanoparticles, the cytotoxicity of DNA-block-copolymer micelles loaded with the widely used anticancer drug Doxorubicin (Dox) was investigated. Dox is known to have side effects such as cardiotoxicity and myelosuppression, therefore targeted delivery is vital.[7] The preparation of Dox-loaded micelles and the determination of loading content were carried out according to the literature (Fig. 1b).[36] The drug payload was 5.6 % of the nanoparticle by weight. The viability of Caco-2 cells after 24 h incubation with Dox-loaded DNA-block-copolymer micelles was compared with several control experiments. The percentage of surviving cells was acquired using an XTT cell proliferation assay. Figure 3A shows that Caco-2 cells incubated with Dox-loaded micelles equipped with targeting units (on average 28 FA on the surface) had a

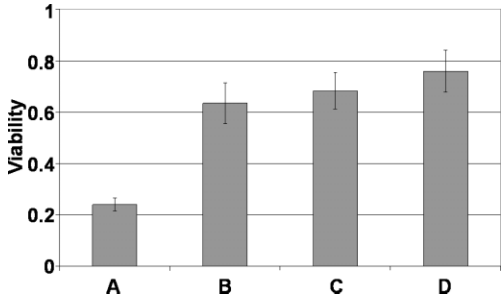

Figure 3. The viability of cells after incubation with A: Dox-loaded micelles covalently linked to targeting unit, B: Dox-loaded micelles with but not covalently linked to folic acid, C: Dox-loaded micelles, D: folic-acid-conjugated micelles in the absence of Dox.

viability of (24.1±2.5) %. The controls consisted of Dox-loaded micelles in the presence of nonconjugated FA (Fig 3B), Dox-loaded micelles in the absence of any targeting unit, (Fig. 3C) and folic-acid-conjugated micelles in the absence of Dox, (Fig. 3D) with viabilities of (63.5±7.9) %, (68.3±7.1) %, and (75.9±8.2) %, respectively. The cell mortalities of the control experiments were significantly lower than when the Dox-loaded micelles were outfitted with FA units, which strongly indicates efficient drug delivery into the tumor cells by the DNA-block-copolymer micelles with the aid of targeting moieties and thus the significant cytotoxicity of these nanoparticles.

Although block copolymers have already been employed for drug-delivery purposes,[37,38] we believe that the nucleic acid/polymeric-hybrid materials presented here represent a significant advantage in the field for several reasons. The DNA-*b*-PPO block copolymers that were synthesized in a

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fully automated fashion were structurally well-defined because the biological segment was monodisperse and contained defined end groups. Such a highly defined structure is an important criterion for approval of a drug or a delivery system. Likewise, the resulting spherical micelles exhibited a narrow size distribution with dimensions in the range of 10 nm. In this regime delivery is independent of the compromised leaky vasculature of the tumor tissue. Most important, however, is the convenience of functionalizing these DNA-block-copolymer nanoparticles. Different amounts of targeting and reporter groups can be incorporated simultaneously at distinct positions on the nanoparticle by hybridization. A variety of 5'- and 3'-modified ODNs bearing different functional groups are commercially available allowing several coupling strategies for a wide range of ligands. In contrast, functionalization of conventional block copolymers with targeting moieties is demanding often requiring multistep synthesis and separation of ligand-modified from ligand-unmodified polymers.[39–41] Moreover, by employing negatively charged DNA with a persistence length of 50 nm as the hydrophilic block, surface exposure of the targeting moieties is guaranteed because, as is well accepted, the polymer chains of the corona in polyelectrolyte block copolymer aggregates are well ordered and completely stretched.[42] When FA is conjugated to other block copolymer systems, e.g., exhibiting a corona of polyethylene glycol, this is not guaranteed to the same extent.[40]

In summary, a novel micelle platform consisting of amphiphilic DNA block copolymers was introduced for chemotherapeutic drug delivery, allowing for combinatorial testing of the drug carrier system. Prior to the investigation of the DNA-block-copolymer micelles, the presence of folate-binding proteins in the cancerous cell line was confirmed and expression levels of three associated genes determined. The corresponding ligand-conjugated ODNs were introduced into the micelles as targeting units via hybridization. The incorporation of fluorescent reporter groups by the same procedure revealed that receptor-mediated endocytotic uptake of the nanoparticles with a diameter of approximately 10 nm was most efficient when the maximum number of ligands was present on the rim of the micelles. Loading Dox into the hydrophobic interior of the ligand-containing micelles resulted in efficient cytotoxicity and high mortality among the cancerous cells. Further studies will investigate targeting with different combinations and ratios of ligands as well as the incorporation of various hydrophobic cancer drugs into the DNA-block-copolymer micelles. Their potential as an anticancer drug-delivery vehicle for in vivo experiments will also be assessed.

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